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INVESTIGATION OF THE ANTAGONISTIC ACTIVITY OF SECONDARY METABOLITES OF PROPIONIC ACID BACTERIA

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Abstract. The practical value of the culture liquid of probiotic bacteria was demonstrated. The culture fluid contains the products of vital activity of probiotic bacteria. It is the product of waste in the manufacture of classical probiotics. The culture liquid can be used to create metabiotics, due to the content of valuable exometabolites in its composition. The results of data on the positive effect of various amounts of culture liquid of propionic acid bacteria on the growth of bifidobacteria were presented. Microbiological studies have shown that 2 – 3 % of the filtrate was effectively reflected in the accumulation of biomass of bifidobacteria. The antagonistic activity of the culture fluid filtrate 2 – 3 % of the strain *P.shermanii*-4 against opportunistic (*Escherichia coli*-ATCC 25922, *Staphylococcus aureus* -ONU-223, *Bacillus cereus*-ATCC 11778) and pathogenic microorganisms (*Salmonella enteritidis* - ONU-466) was studied. It was found that the use of a 2 % culture supernatant inhibits the growth of all opportunistic microorganisms, in addition to the pathogenic strain *Salmonella enterica*-ONU-466. With an increase in the dose of the filtrate, a small delay in the growth of *Salmonella enteritidis*-ONU-466 and an increase in the sensitivity of opportunistic microorganisms were observed.

Key words: culture liquid, supernatant, propionic acid bacteria, metabiotic.

ДОСЛІДЖЕННЯ АНТАГОНІСТИЧНОЇ АКТИВНОСТІ ВТОРИННИХ МЕТАБОЛІТІВ ПРОПІОНОВОКИСЛИХ БАКТЕРІЙ

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Анотація. У статті наведено результати досліджень стосовно позитивного впливу різної кількості культуральної рідини пропіоновокислих бактерій на збільшення біомаси *Bifidobacterium bifidum-1*. Мікробіологічні дослідження дозволили визначити, що кількість фільтрату від 2 % до 3 % ефективно відображається на накопиченні біомаси біфідобактерій. Вивчено антагоністичну активність фільтрату культуральної рідини штаму *Propionibacterium shermanii-4* у кількості від 2 % до 3 % по відношенню до умовно-патогенних (*Escherichia coli*-ATCC25922, *Staphylococcus aureus*-ОНУ-223, *Bacillus cereus*-ATCC11778) та патогенних мікроорганізмів (*Salmonella enteritidis*-ОНУ-466). Встановлено, що використання мінімальної концентрації 2 % супернатанту культуральної рідини пригнічували ріст усіх умовно-патогенних мікроорганізмів, окрім патогенного штаму *Salmonella enterica* -ОНУ-466.

Ключові слова: культуральна рідина, супернатант, пропіоновокислі бактерії, метабіотик.

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Introduction

Various adverse effects of exogenous and endogenous nature on the human body cause changes in the immune response and can affect the qualitative and quantitative composition of the normal microbiota of the gastrointestinal tract (GIT). Microbiocenosis is an evolutionary system that is based on the principle of self-regulation. Pathological changes in symbiotic microflora in most cases are restored independently. In clinical practice, treatment with the appointment of antibiotics is quite widespread, which is often the standard solution for various infectious diseases of bacterial etiology. Unfortunately, after the irrational use of antibiotics, microbiota, GIT can not recover independently. Thus, the search for adequate correction of intestinal microbiocenosis disorders in patients receiving antibiotics is an actual problem.

Formulation of the problem

Probiotics based on living microorganisms (bifidobacteria, lactobacilli, propionic bacteria, etc.), prebiotics (indigestible substances stimulating the activity of certain microorganisms, symbiotics (combinations of probiotics and prebiotics), as well as metabolic probiotics – metabiotics (microbial metabolites) are presented in the arsenal of medicines aimed for the restoration of normal microbiota [1].

However, the therapeutic and prophylactic effect of using probiotics is not always achieved. One of the

main reasons for the low effectiveness of probiotics is that the strains included in their composition, like any other microorganisms, enter into a symbiotic relationship with the human microbe and may be rejected due to bio-compatibility.

It is shown that microorganisms-probiotics and autochthonous microorganisms can both stimulate and inhibit the growth and antimicrobial properties of each other. According to the analysis of experimental data [2], probiotic bacteria are divided into: biocompatible with the dominant microbiotic of the individual and capable of inhibiting the growth of pathogens; Non-biocompatible with normobiota, but capable of stimulating its protective properties and provide antagonism to pathogenic and opportunistic microorganisms; Non-biocompatible is able to inhibit the antimicrobial properties of the autochthonous microbioma of the gastrointestinal tract and have a weak antagonistic activity against pathogen.

To determine the biocompatibility of probiotic preparations with autochthonous microflora, it is necessary to conduct a series of studies for a specific person or to develop autobiotics, which is a laborious process. In this regard, scientists have high hopes for a metabiosis that does not contain microorganisms, and lack the disadvantages mentioned above.

Literature review

Attention to classical propionic acid bacteria as potential probiotics conditioned their properties to produce antibiotic and bifidogenic substances of different nature. The antibiotic activity of propionic acid bacteria against gram-positive and gram-negative bacteria, yeast, fungal mycelium has been proven. Currently, it is believed that it proteazoaktyvuyuchi polypeptides are very similar in structure to peptides isolated from *Lactobacillus lactis*. Their gene contained in plasmids and is characterized by horizontal transfer. Antibiotic activity inherent *Propionibacterium jensenii*-SM11 and different strains of *Lactobacillus paracasei subsp. paracasei* to yeast that cause spoilage of dairy products [3].

Bifidogenic activity of propionic acid bacteria is caused by production of 1,4-hydroxy-2-naftoynoyi acid, 2-amino-3-carboxy-1,4-naphthoquinone and somewhat less short-fatty acids, which both are inhibitors of Gram-negative facultative and obligate anaerobes. The main producers of bifidogenic factors are bacteria *Propionibacterium freudenreichii subsp. freudenreichii* and *P. freudenreichii subsp. Shermanii* [4,5]. Also revealed synthesis bifidogenic factors by *P. acidipropionici* under aerobic conditions. It was interesting to investigate the similar effect of the culture liquid that was obtained by culturing propionic acid bacteria.

Culture fluid is a waste product in the traditional production technology of bacterial preparations or concentrates. It contains products metabolism of probiotic bacteria and can be used as the basis un-cellular form probiotic. Utilisation of culture fluid as waste caused to significant economic losses and environmental pollution [6]. Therefore, the development and production of cell-free probiotics is one of the stages of creating low-waste production of bacterial preparations – it is an innovative way of biotechnology.

In an experimental study on the influence of culture fluid supernatant lactobacilli and bifidobacteria and their inactivated form of the cell revealed that they have different rates of recovery of the intestinal microbiota. Inactivated probiotic bacteria are not reduced severity dysbiotic changes in the colon. Only the supernatant improves almost all indicators of GIT microbiota [7,8].

This shows that the effectiveness of probiotic preparations based by the waste products of microorganisms. In this regard, the urgent task of biotechnology is the development of technology production metabiotics from cell-free filtrates or supernatant.

Determination of the antagonistic activity of *Propionibacterium shermanii* – 4

The aim of this study was to study the antagonistic effect of the culture fluid *Propionibacterium shermanii*-4 on the autochthonous microbiota of the gastrointestinal tract and opportunistic microorganisms.

The museum strains from the department of biochemistry, microbiology and physiology food ONAFT *Bifidobacterium bifidum*-1, *Propionibacterium shermanii*-4 were used for our experimental work. Opportunistic pathogens (*Escherichia coli*-ATCC25922, *Staphylococcus aureus*-ONU-223, *Bacillus cereus*-ATCC11778) and pathogens (*Salmonella enteritidis*-ONU-466) were used as test-strains.

The daily culture *Bifidobacterium bifidum*-1, after pre-cultivation for 24 hours at temperature (37 ± 1) °C was used as representative of normal gastrointestinal microbiota man.

Incubation of *P*.shermanii-4 was performed during 24 hours at the temperature (30 ± 1), which was at least 1 10^9CFU/cm^3 .The supernatant culture fluid of *P.shermanii*-4 was obtained by centrifugation at 10,000 min^{-1} for 15 minutes. Culture fluid was filtrated through bacterial filters (Millipore, 0,22 um) under aseptic conditions.

The resulting filtrate culture fluid of the strain *P.shermanii*-4 was inoculated in an amount from 1 – 3 cm^3 in the lactose medium, followed by culturing for 24 hours at the temperature (30 ± 1) °C for testing absence of viable cells in culture fluid filtrate propionic acid bacteria. Accounting the results was made by direct counting of propionic acid bacteria in the counting chamber of Horyaev.

Bacteria cells of propionic acid bacteria were not found in anything experimental microscopy sample.

The cultivation of *B.bifidum*-1 with addition the filtrate culture fluid *P.shermanii*-4 was performed on lactose medium with soy whey for 24 hours at the temperature (37 ± 1) °C to study the impact of the supernatant culture fluid of *P.shermanii*-4. The daily culture of B.bifidum-1 was standardized to $1 \cdot 10^6$

CFU/cm^3. Value inoculum biomass of bifidobacteria to filtrate culture fluid propionic acid bacteria were as follows: 1:1; 1.5:1; 2:1; 2.5:1; 3: 1. In the control sample, filtrate culture fluid of propionic acid bacteria was replaced by distilled water in equal extent.

Quantifying viable cells of bifidobacteria in experimental and control samples was carried out by tenfold dilutions in test tubes with lactose environment. Incubation of cultures was carried out at the temperature (37 ± 1) °C for 72 hours.

To study the formation of propionic acid bacteria specific antimicrobial compounds used method [9]. Petri dishes with 25 cm^3 IPA seeded lawn relevant test cultures at concentrations – 10^6 CFU/cm^3 standard turbidity Mc Farlanda. It was kept 1 hour at the temperature (37 ± 1) °C. Holes were made by the special sterile drill bit diameter 8 mm in medium. The supernatant of in an amount of 80 cm^3 was filled hole in the nutrient medium. Cups were held at the temperature (5 ± 1) °C for 2 hours (for the diffusion of culture broth), and incubated under anaerobic conditions for 18 hours at the temperature (37 ± 1) °C. The results were evaluated by measuring the area of suppression growth.

In our investigation found that a positive impact on increasing the biomass *B. bifidum*-1 was seen in all experimental samples after culturing at (37 ± 1) °C for 24 hours on a lactose medium with addition different amounts of supernatants culture fluid *P. shermanii*-4. An increasing number of filtrate culture fluid *P. shermanii*-4 caused the increasing the number of viable cells of B. *bifidum*-1 (Table 1.)

Table 1 - Effect of secondary metabolites *P.shermanii-4* on the growth of *B. bifidum-1*

№ experimental sample	Proportion inoculum of *P.shermanii-4* and *B. bifidum-1*	Number of viable cells of *B. bifidum-1*, CFU/cm^3
1	1:1	$3\cdot10^9$
2	1:1,5	$5\cdot10^9$
3	1:2	$9\cdot10^9$
4	1:2,5	$1\cdot10^{10}$
5	1:3	$2\cdot10^{10}$
control	1:0	$1\cdot10^9$

The next stage of our study was to investigate the antagonistic activity of the supernatant culture fluid *P.shermanii*-4. The supernatant in an amount of 2 %, 2.5% and 3 % was used for the manifestation of antagonistic activity against pathogenic and opportunistic strains. This choice is due to the previous experiment described above.

The increase of number of filtrate culture fluid propionic acid bacteria caused the increase sensitivity of opportunistic pathogenic strains (Table 2, Fig.1). The greatest sensitivity *Bacillus cereus*-ATCC11778 and in *Staphylococcus aureus*-ONU-223 were found to secondary metabolites *P.shermanii*-4.

Table 2 - Antimicrobial activity of the supernatant culture fluid *P.shermanii*-4

Amount of supernatant, %	Zone of inhibition of growth of test cultures, mm			
	Bacillus cereus-ATCC11778	*Staphylococcus aureus*-ONU-223	*Escherichia coli*-ATCC25922	*Salmonella enteritidis*-ONU-466
2	13±0,5	16±0,5	9±1	-
2,5	15±1	17±1	10±0,5	-
3	16±1	19±1	12±1	2±1

After introduction of the filtrate culture fluid propionic acid bacteria and bifidobacteria biomass in a ratio of 1:1 number of viable cells of bifidobacteria was increased to $3\cdot10^9$ CFU/cm^3. This result was within the meaning of the control sample – $1\cdot10^9$ CFU/cm^3 – where the supernatant was replaced by saline in equal volume. In the second experimental sample where inoculums were used in a ratio of 1:1.5 there were no significant changes, biomass *of B. bifidum*-1 increased only to $5\cdot10^9$ CFU/cm^3. However, the third experimental sample where the inoculums proportion was 1:2, there was a significant increase in viable cells compared with controls – $9\cdot10^9$ CFU/cm^3. With further increase in the dose of filtrate culture fluid *P. shermanii-4* biomass of *B. bifidum-1* continued to grow, but not as intense, at the proportion of 1: 2.5 – were increased to $1\cdot10^{10}$ CFU/cm^3, the proportion of 1:3 – were increased to $2\cdot10^{10}$ CFU/cm^3

The results of the microbiological results, we recommend filtrate *P. shermanii-4* in an amount of 2 % to 3 %, as growth promoter of bifidobacteria.

Propionic acid bacteria are known for their ability to produce propionic acid, which because of its antimicrobial and antifungal activity is used as a protection against microbial spoilage of food products. To secondary metabolites of propionic acid bacteria belong bacteriocins, represented by antimicrobial peptides or proteins, which exhibit antagonistic activity of pathogenic and opportunistic strains [10,11]. Growth of *Escherichia coli*-ATCC25922, *Staphylococcus aureus*-ONU-223, *Bacillus cereus*-ATCC11778 was inhibited in the presence of a *P.sheranii-4* culture fluid filtrate. This can be explained by the fact that antagonistic activity is due to the accumulation of antimicrobial substances in the culture fluid of *P.sheranii*-4. Accumulation of these antimicrobial substances in the culture liquid allows, to use it as a raw material for creating a probiotic of the metabolic type.

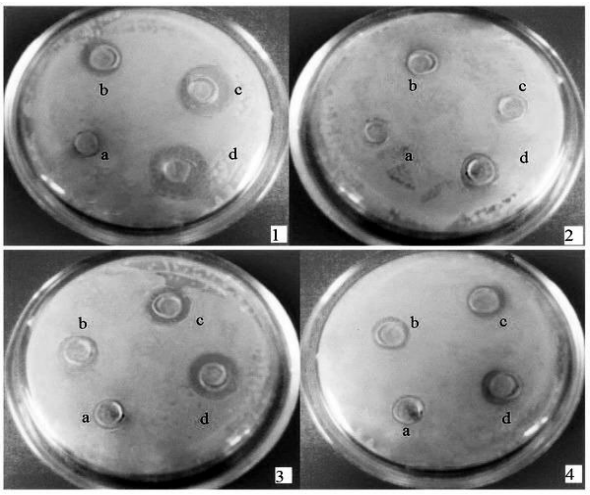

Fig.1. Antagonistic effect of different concentrations of the supernatant culture fluid *P.shermanii* -4 on the growth of test-strains:
1) the inhibition zones of growth of *Staphylococcus aureus*-ONU-223 using bacterial supernatant in such concentrations: a – control, b – 2 %, c - 2.5 %, d – 3 %; 2) the inhibition zones of growth of *Salmonella enteritidis*-ONU-466 using bacterial supernatant in such concentrations: a – control, b – 2 %, c - 2.5 %, d – 3 %; 3) the inhibition zones of growth of *Bacillus cereus*-ATCC11778 using bacterial supernatant in such concentrations: a – control, b – 2 %, c - 2.5 %, d – 3 %; 4) the inhibition zones of growth of *Escherichia coli*-ATCC25922 using bacterial supernatant in such concentrations: a – control, b – 2 %, c - 2.5 %, d – 3 %

The results of the studies prove the expediency of using secondary metabolites of propionic acid bacteria.

These metabolites can be used as an independent acellular probiotics and as the growth stimulators for the normal microbiota of the human gastrointestinal tract. In connection with this, our next research will be devoted to the determination of prebiotic components of the non-carbohydrate nature of propionic acid bacteria.

Conclusion

As a result of the research, it was found that the culture liquid of the strain *P.shermanii*-4 was capable of stimulated the growth of bifidobacteria. The recommended amount of culture fluid filtrate is from 2 % to 3 %The number of viable cells was increased to $2 \cdot 10^{10}$ CFU/cm^3

With the selected concentrations, antagonistic activity against pathogenic and opportunistic strains was investigated. It was found that the use of a minimum concentration in the amount of 2 % supernatant of the culture liquid suppressed the growth of all opportunistic microorganisms (*Escherichia coli*-ATCC25922, *Staphylococcus aureus*-ONU-223, *Bacillus cereus*-ATCC11778). However, when using a 2 % culture fluid filtrate, antagonistic activity to the strain *Salmonella enteritidis*-ONU-466 was not appeared.

The sensitivity of opportunistic strains increased, when the amount of the filtrate was increased to 3 %. This was indicated by an increase in the zone of inhibition of growth of test cultures. There was also a slight delay in the growth of *Salmonella enteritidis*-ONU-466. The growth inhibition zone of this strain was 2 ± 1 mm.

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ИССЛЕДОВАНИЕ АНТАГОНИСТИЧЕСКОЙ АКТИВНОСТИ ВТОРИЧНЫХ МЕТАБОЛИТОВ ПРОПИОНОВОКИСЛЫХ БАКТЕРИЙ

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Аннотация. В статье приведены результаты данных о положительном влияния разного количества культуральной жидкости пропионовокислых бактерий на увеличение биомассы *Bifidobacterium bifidum-1*. Микробиологические исследования позволили определить, что количество фильтрата от 2 % до 3 % эффективно отражается на накоплении биомассы бифидобактерий. Изучена антагонистическая активность фильтрата культуральной жидкости штамма *Propionibacterium shermanii-4* в количестве от 2 % до 3 % по отношению к условно-патогенным микроорганизмам (*Escherichia coli*-ATCC25922, *Staphylococcus aureus*-ОНУ 223, *Bacillus cereus*-ATCC11778) и патогенным микроорганизмам (*Salmonella enteritidis* - ОНУ-466). Установлено, что использование минимального количества 2 % супернатанта культуральной жидкости подавляли рост всех условно-патогенных микроорганизмов, кроме патогенного штамма *Salmonella enterica*-ОНУ-466.

Ключевые слова: культуральная жидкость, супернатант, пропионовокислые бактерии, метабиотик.

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